STUDIES ON BIOSYNTHESIS OF MONOIODOTYROSINE

1. Purification and Properties of the Enzyme

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The studies of the enzyme catalysing the biosynthesis of monoiodotyrosine (MIT) were made by Fawcett and Kirkwood[1,2] with the cell-free system and by Taurog et al.[3] with the subcellular fraction. Recently the relation between two systems was reported by Serif and Kirkwood.[4] In order to clarify the properties of the enzyme and the mechanism of iodination to be induced by it. It is desirable for the enzyme to be isolated in pure state. But no report concerning its purification has been available.

All of the various attempts to purify this enzyme have been unsuccessful till the discovery of the fact that it consisted of a macromolecular protein (apoprotein) and a dialyzable anionic substance (cofactor). These two components were obtained from the extract of the submaxillary tissue by column chromatography with hydroxylapatite.[5] The enzyme exhibits catalytic activity only when both components are present. An attempt to purify them separately is proved successful, and the apo-protein was obtained from the bovine submaxillary tissue and the cofactor from the bovine thyroid tissue almost pure state. The present paper deals chiefly with the method of purification and the properties of the purified substances and pronounced following view.

1. The enzyme system contains neither peroxidase[6,7] nor xanthine oxidase[8] which have been considered to be responsible for the oxidation of iodide in the thyroid.

2. The cofactor is not identical with the flavin cofactor discovered by Tong et al.[9] that stimulates the iodinating system which presents in mitochondrial-microsomal fractions isolated from sheep thyroid glands.

EXPERIMENTAL

Measurement of Enzyme Activity

1. *Incubation proceduers*----The incubation system for the measurement of enzyme activity contained the following constituents: 0.1 ml of apo-protein solution, 0.1 ml of cofactor solution, 0.05 ml of 10^{-5}M potassium iodide solution, 0.2 μc ^{131}I, 0.1 ml of 2×10^{-2}M tyrosine solution, and 0.1 ml of 0.2M sodium phosphate buffer, pH 7.4. The reaction was stopped with addition of 0.1 ml of 0.1M sodium thiosulfate. Cupric ion was not always supplied. Portions of the incubated reaction mixtures were then analyzed directly by paper chromatography.

2. *Chromatographic analysis*----All chromatographic separations were carried out on Toyo filter paper No. 50. The general techniques were carried out through the method of Fawcett and Kirkwood.(1) The chromatographic solvent was the mixture of butanol-acetic acid-water in the volume proportions of 4: 1: 2. The ^{131}I in each components were determined by cutting out the zones of activity on the chromatograms and counting them directly in a well type scintillation counter. The radio-activity in each components were expressed as a percentage of the total ^{131}I present on the chromatogram. The enzyme activity was indicated as the sum of free MIT and iodinated protein fraction on the origin.

Purification of Apo-protein

In early investigation to purify this enzyme, the enzyme activity was diminished following the progress of purification in various methods. Although the enzyme activity of tissue homogenate was not decreased by dialysis against 0.1 M sodum phosphate buffer, pH 7.4, for 24 hours at 3°C, the enzyme purified by acetone or ammonium sulfate fractionation was greatly inactivated by dialysis in the same condition. From this fact it was presumed that some small molecular substance was contained in the enzyme system. As we found, the enzyme activity was clearly recovered by combining the two far separated fractions which were eluted from hydroxylapatite column, and it became possible to purify each of the two components of the enzyme.

The effective component of the first fraction was a dialyzable organic compound, and assumed to be the cofactor of this enzyme system. One of the fourth fraction was protein, and it was inferred to be the apo-protein. (The results of stepwise elution and enzyme activity of the mixtures of the fractions were illustrated in Fig. 1.) Bovine submaxillary tissues were used, as the material to obtain the apo-protein, because thyroid homogenate was impossible to treat without disapperance of activity. Unless otherwise indicated all steps were carried out at temperature of 4°C. All reagents were made up in glass-distilled water.

1. *Extraction*----Fresh bovine submaxillary tissues were obtained at a local abattoir, chilled immediately, packed in ice, and transported to the laboratory as quickly as possible. The tissues were freed of extraneous tissues, minced with scissors, and then homogenized in batches, each with 2 volumes of 0.05 M sodium acetate, in prechilled, small scale Waring Blendors, at full speed

for 1 minute. The suspension was centrifuged for 10 minutes at 800×g and the supernatant fluid was collected. (Fraction I).

2. *Calcium Phosphate Gel Adsorption and Elution* ---- 400 ml of freshly prepared calcium phosphate gel (12 mg/ml) was added to 800 ml of Fraction I in order to adsorb the apo-protein. The mixture was stirred for 5 minutes, and then centrifuged. The supernatant fluid was discarded and the gel washed with 400 ml of 0.2M sodium phosphate buffer, pH 7.4. The gel was then eluted twice with 300 ml portions of eluting solution (0.1 M phosphate buffer at pH 7.4 containing 0.3 M ammonium sulfate) in order to remove the apo-protein, and elutants were combined (Fraction II).

3. *Ammonium Sulfate Precipitation* ---- This process was taken to condense rather than to fractionate the apo-protein.

With constant, rapid stirring 168 gm of finely powdered ammonium sulfate were added to 800 ml of Fraction II. After being left as it was for 20 minutes the mixture was centrifuged for 20 minutes at 2,000×g. The residue was discarded. In a similar manner 144 gm of ammonium sulfate were added to the supernatant fluid. After centrifugation the precipitate were collected and put between several sheets of filter paper and pressed with hand press in order to remove the salts contained in the precipitated solid as much as possible. The solid was dissolved in 20 ml of 10^{-3} M sodium phosphate buffer at pH 6.8, and then centrifuged to remove the refuse of filter paper contaminated in the solid. (Fraction III).

4. *Column Chromatography* ---- Hydroxylapatite was used for chromatographic purification of the apo-protein, wich was prepared according to Tisselius et al.[5] 10 ml of Fraction III was placed on a column (1.5×12cm) of hydroxylapatite equilibrated with 5×10^{-3} M sodium phosphate buffer, pH 6.8. After all solution passed through and the protein was adsorbed completely, the column was eluted by stepwise elution method. For the first step it was eluted with about 150 ml of 0.1 M sodium phosphate buffer, 7.4 containing 0.1 M ammonium sulfate, at a flow rate of 20 ml/hr., until the column was colorless and the extinction value at 280 mμ of last eluting fraction was shown less than 0.1 ($-\log T$). For the second step the column was eluted continually with 0.1 M sodium phosphate buffer, pH 7.4 containing 0.2 M ammonium sulfate. Five milliliter samples were collected by use of a fraction collector. The pattern of eluted activity is shown in Fig. 2.

The active apo-protein exists mainly in the second step. When the second step was contaminated with a colored protein, rechromatography was performed in the same manner. Typical recovery data for the whole process are given in Table I.

TABLE I

Purification of Apo-protein

	Volume of solution ml	Protein content mg/ml	Total protein mg	Enzyme activity %
Crude submaxillary homogenate (Fraction I)	290	15.6	4522	35.4
Gel eluate (Fracton II)	230	3.4	768	78.2
Final preparation	20	0.67	13.4	82.1

The enzyme activity was indicated by the per cent of ^{131}I converted to MIT, which is produced by adding an equal amount of cofactor. Protein contents were measured from N_2 contents determined by microkjeldahl, using Azotometer. (10)

The comparison of enzyme units in each step of purification, in order to indicate the grade of purification, can not be carried out, because the enzyme units have not been measured owing to the following reasons.

1. For protein concentration in reaction mixture an optimum concentration of cofactor is required to obtain the highest enzyme activity. But the methods for the measurement of cofactor contents have not been found, so that the enzyme activity of a certain solution in steady state is not able to be measured.

2. The enzyme activity per protein concentration does not mean the enzyme unit, because it was found that some amounts of contaminating protein inhibit this enzyme reaction. (unpublished data)

Purification of Cofactor

It is possible to purify the cofactor from bovine submaxillary tissues, and the method had been reported in previous papers.(11,12) But it is not easy to obtain the lyophylizing samples from submaxillary tissues, because it is difficult to remove the mucus. While, from thyroid tissues it is easy and is more in yield.

Fresh bovine thyroid tissues were freed of extraneous tissues, minced with scissors, and then homogenized with two volumes of 0.05 M sodium acetate by Waring Blendor. The suspension was centrifuged for 10 minutes at 600×g and the pH of supernatant was adjusted to 4.5 with 1 N acetic acid, and the suspension was allowed to stand overnight at 0-4°C for precipitation of protein. The precipitated solids were removed by centrifugation for 10 minutes at 600×g. About 100 ml of the clear supernatant was added to 200 ml of calcium phosphate gel (12 mg/ml), stirred, and kept still for 5 minutes, and then centrifuged. Almost deproteinized supernatant was adjusted to pH 7.4 in order to precipitate calcium phosphate; The precipitate was discarded by filtration. The filtrate was passed through Dowex 50 (H-type, 100-200 mesh) column (1.5×15 cm) to

remove cations with flow rate 300 ml/hr. The passed solution was applied to Dowex 1×2 (formate-type, 200-400 mesh) column (1.5×18 cm), which was prepared with the method of Potter et al.[13] The elution was carried out with 0.2 M formic acid in 5 ml fraction. 0.3 ml of each fraction was lyophilized, and its activities were determined. Typical recovery data are given in Fig. 3. When the replacement of resin to formate type was incomplete and when the length of column was insufficient, the lyophilization of cofactor was impossible without disappearence of activity. Denaturation occurred by chloride ion eluted together in the former case, and by phosphoric acid in the latter case.

The comparison of activity between the cofactor obtained by above method and the flavin cofactor reported by Tong et al.[9] to activate the mithochondrial-microsomal fraction in thyroid glands, is shown in Table II.

TABLE II

The Comparison of Flavin Mononucleotide and Isolated Cofactor with Purified Apo-protein

Added cofactor	Final molar concentration	Conversion of ^{131}I to MIT
		%
Isolated cofactor	—	75.5
〃 added cupric ion	—	84.2
Flavin mononucleotide	10^{-3}	32.1
〃 added cupric ion	10^{-3}	0.9
Flavin mononucleotide	10^{-4}	6.5
Nothing	—	1.1

In these experiments the reaction mixture was incubated for 30 minutes at 37°C.

Properties of Apo-protein

1. *Stability*

The apo-protein is stable at low temperature over the range of pH 4.0～8.2. The activity is diminished by heating at 70°C for 10 minutes, but the loss at 60°C for 10 minutes is hardly detectable.

If the purified apo-protein is lyophilized, the enzyme activity is completely lost. Activity is lost when the protein is kept dry in a desiccator. The suspension precipitated by ammonium sulfate was kept without inactivation at −18°C for several weeks.

2. *Paper Electrophoresis*

Since not enough material was available for a moving boundary electrophoresis, a paper electrophoretic pattern was determined with the use of diethylbarbiturate buffer at pH 8.6, ionic strength 0.05. The results shown in Fig.

4. demonstrate the effective separation of the apo-protein from contaminating proteins by hydroxylapatite chromatography.

3. *Ultracentrifuge Examination*

The purity of the apo-protein was examined with the Phyue ultracentrifuge. The solution contained 0.5 per cent apo-protein in phosphate buffer, pH 6.8, ionic strength 0.1. A single, symmetrical peak was observed, which moved with a velocity corresponding to a sedimentation constant $S_{20,w}$ 6.12 Svedberg units.

4. *Absorption Spectrum*

The visible and ultraviolet absorption spectrum of a highly purified apo-protein exhibits only a absorption maximun at 280 mμ, so that the presence of peroxidase, which is a heme protein, can be denied.

Properties of Cotactor

The amount of cofactor obtained in a solid and pure state is only a trace, so that analysis of the chemical properties has not been performed yet. But the properties observed in a process of purification are described below.

1. It is inactivated by heating in aqueous solution at 80°C for 10 minutes. Furthermore, it is gradually inactivated by keeping in store at 4°C and within a week its loss is complete.

2. It does not seem to have an absorption at 260 mμ, because the active fraction in its rechromatography exhibits no extinction.

3. It seems to be an anionic compound, because it passed cation exchange resin without losing activity and adsorbed in anion exchange resin.

DISCUSSION

The isolated apo-protein behaves in a hydrophobic way toward the solution used for the staining after the paper electrophoresis and suffers denaturation when it is lyophilized and its nitrogen content is only 14.21 per cent. Therefore it is thought to be a lipoprotein with a pretty high lipid content.

The apo-protein, whose method of purification and properties were described in this paper, is the one extracted from bovine submaxillary tissues, and since it is confused with cofactor extracted from the thyroid tissues in the study of the reaction mechanism of the biosynthesis of MIT, it is a crucial question whether the thyroid tissue contains an apo-protein of the same kind or not. Though it cannot be asserted with full confidence that the two apo-proteins have identical functions until the apo-protein contained in the thyroid tissue is isolated, some experimental results suggesting the affirmative answer to this question was reported by the author's coworker, Iwakura. (unpublished data). He showed, by immunochemical analysis employing the agar gel double diffusion method, that antiserum against the submaxillary apo-protein gave the precipitation lines with crude enzyme protein of the thyroid. This means that the thyroid contains an immunologically same kind of protein and

therefore probably an apo-protein is similar to that contained in the submaxillary tissue.

The techniques for extracting the apo-protein from the submaxillary tissue cannot be used for the thyroid, because, while the submaxillary homogenate does not show detectable decrease of enzymic activity after standing overnight at low temperatures, the thyroid homogenate loses all the enzymic activity under the same condition. This fact seems to indicate that the latter contains some factor, perhaps a proteinase, which inactivates the apo-protein Thus it appears that successful purification of the thyroid apo-protein depends on the discovery either of a quicker method of purification or some way of removing the inactivating factor.

Neither the purified apo-protein nor the purified cofactor shows enzymic activity by itself. The activity is shown only when both are present. Addition of an extremely minute quantity of the isolated cofactor results in a high enzyme activity. Tong et al.[9] has reported that addition of flavin cofactor to the cell-particulate fraction of the thyroid increases the formation of iodoprotein. When flavin cofactor is used as substitute for isolated cofactor in this reaction, its results are shown in Fig. 4. At a concentration of 10^{-3} M flavin mononucleotide gives about one third of the activity given by the isolated cofactor, and at lower concentration hardly any at all.

Furthermore, the difference in this reaction is pointed out when the isolated cofactor or flavin mononucleotide is used. Addition of cupric ion causes increase of the activity in the latter case. The optimum pH value is between 7.4 and 7.8 in the former and between 9 and 10 according to Tong et al. in the latter.

These results do not seem to indicate that the flavin cofactor is a natural cofactor for iodoprotein formation. Moreover, the isolated cofactor does not show the absorption spectrum and fluorescence characteristic of flavin even at concentrations high enough for exhibition of enzyme activity. Therefore it is not conceivable that the isolated cofactor belong to the flavin compound. This does not mean, of course, the fact should be ignored that the flavin cofactor has the power to induce the enzyme activity to a certain degree.

In the formation of MIT in the thyroid, it is advocated that iodide is first oxidized into highly reactive elemental iodine.[15]

$$2I^- \text{-------} I_2 + 2e$$

The factor that catalyzes the reaction is thought to be peroxidase or xanthine oxidase. The enzyme system obtained in the pure state by the author, however, contains any heme neither in the apo-protein nor in the cofactor. This fact deny the possibility of contamination of enzyme system by peroxidase, which is a heme-protein. In the other hand, if it contains xantine oxidase, its action ought to be inhibited by cupric ion. The fact that the activity is increased by the addition of cupric ion denies the possibility of the presence of xanthine oxidase. Admitting the absence of peroxidase and xanthine oxidase, it is impossible to say whether there is any

other enzyme possessing the ability of catalyzing the reaction, because no means is available at present for determing if the elemental iodine is produced by this enzyme system. It is a moot question whether elemental iodine must be produced before MIT formation or there is any other way of biosynthesis of MIT.

SUMMARY

1. The purification of the monoiodotyrosine synthesizing enzyme system is described. This system can be divided into two parts: a macromolecular protein (apo-protein) and a dialyzable anionic substance (cofactor).

2. The apo-protein has been purified from bovine submaxillary tissues by chromatography on hydroxylapatite column. Paper electrophoresis and ultracentrifugation are made to determine the homogeneity of the preparation.

3. The cofactor has been purified from bovine thyroid tissues by chromatography on Dowex 1×2 column.

4. It is discussed that this enzyme system does not contain peroxidase or xanthine oxidase which is believed to participate in oxidation of iodide.

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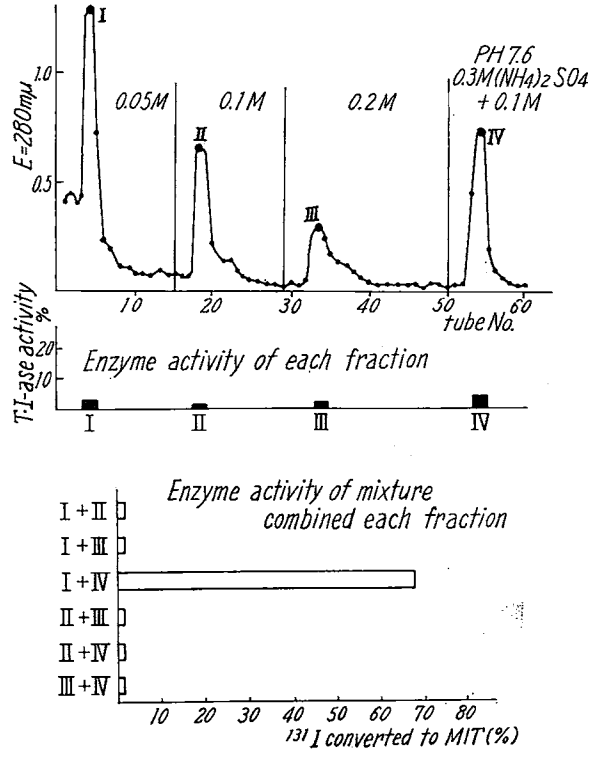

Fig. 1. Chromatography of submaxillary extract on 1.5×12 cm column of hydroxylapatite.

The eluting solution was 0.05 M sodium phosphate buffer at pH 6.8 for the first step, 0.1 M of the same buffer for the second step, 0.2 M of the same buffer for the third step and 0.1 M sodium phosphate buffer for the third step and 0.1 M sodium phosphate buffer at pH 7.4 containing 0.3 M ammonium sulfate for the fourth step. A volume of 4-6 ml was collected in the tube at an average flow fate of 15 ml/hr. The enzyme activity of each fraction and mixture of fractions were determined.

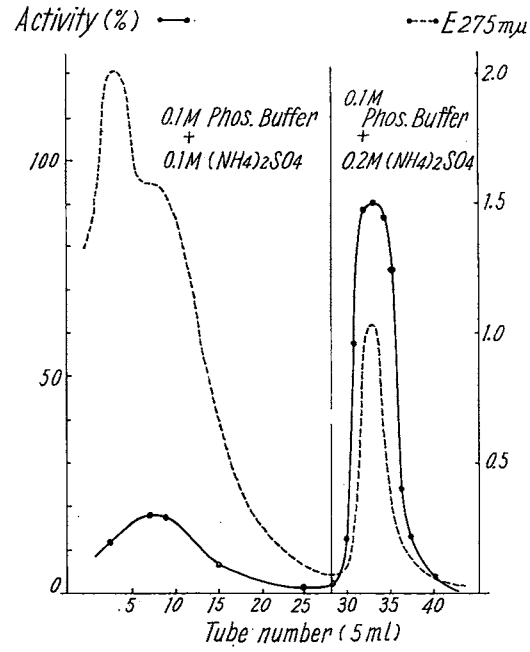

Fig. 2. Chromatography of apo-protein on 1.5×12 cm column of hydroxylapatite.

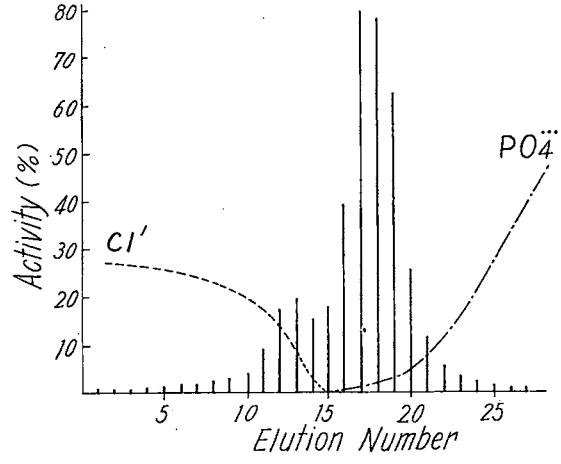

Fig. 3. Column chromatography of thyroid extract on Dowex 1 × 2-formate

Column dimensions were 1.5×18 cm and eluting solution was 0.2 N formic acid. A volume of 4-6 ml was collected in the tube at an average flow rate of 40 ml/hr. Cl ion was detected by addition of $AgNO_3$. PO_4^{---} ion was determined by the method of Allen.[14]

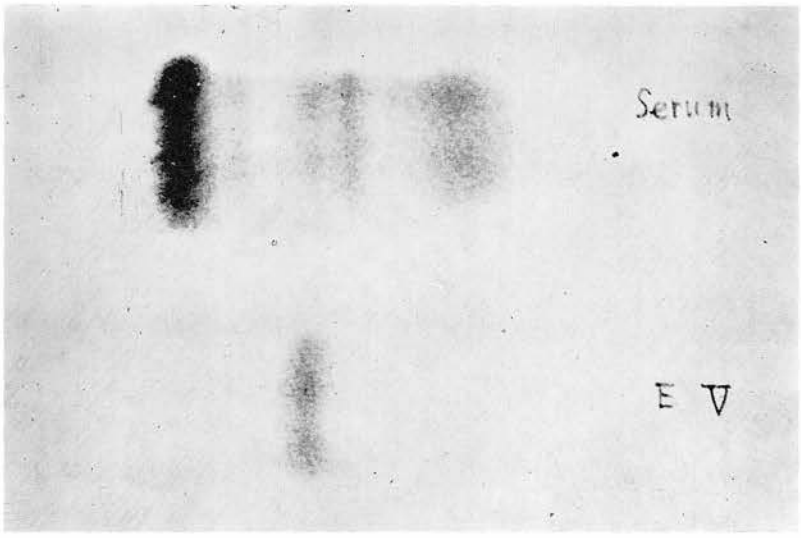

Fig. 4. Paper electrophoretic pattern of the purified apo-protein.

Veronal buffer pH 8.6, ionic strength, 0.05 was used. The site of pattern was compared with human serum which moved on the same paper.

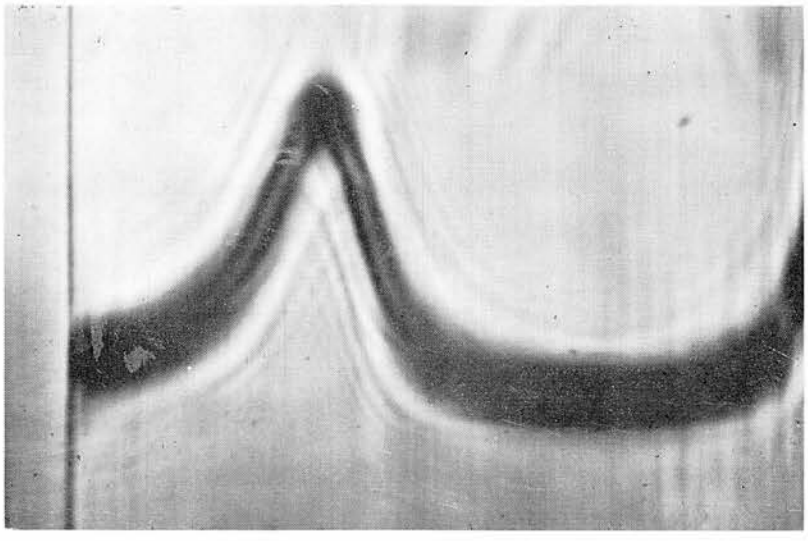

Fig. 5. Sedimentation pattern of the purified apo-protein.

Concentration of protein in phosphate buffer, pH 6.8, ionic strength 0.1 : 0.5 per cent. Bar angles : 55°. Photographs at 52 minutes after rotor reached speed of 50,000 r. p. m. Direction of sedimentation is to the right.